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PATENT SPECIFICATION

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COMPLETE SPECIFICATION

Method for the Treatment of Malignant Tumors

We, MERCK & CO., INC., a body corporate organised and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates generally to methods for treating malignant tumors in vertebrates. More particularly, it concerns a new method of reducing the weight, and thus the size, of tumors in vertebrates. Still more specifically, it is concerned with a method for substantially improving and increasing the oncolytic effect of spores of non-pathogenic, anaerobic strains of Clostridia. Still more precisely, the invention involves a method for treating malignant tumors in vertebrates by combined therapy with spores of non-pathogenic, anaerobic Clostridia and chemotherapeutic agents.

Möse and Möse have reported that certain non-pathogenic, anaerobic Clostridia cause extensive liquefaction and lysis of tumors in vertebrates. Zeitschrift für Krebsforschung, *63*: 63—74 (1959); *63*: 447—455 (1960). Such oncolysis, with consequent reductions in weight and size of the tumor, is achieved by injection of spores of the Clostridia into the tumor-bearing vertebrate. The spores localize and germinate in tumor tissue but not in normal tissue. In tumors of sufficient size liquefaction of substantial amounts of viable tumor tissue occurs, leading to a significant reduction in tumor weight. The spores are usually administered intravenously in physiological saline, although other parenteral routes of administration such as the intramuscular or intraperitoneal routes and other physiologically acceptable vehicles such as aqueous glucose or Ringer's solution may be employed.

The spore preparations are obtained by cultivation of non-pathogenic strains of Clostridia. Examples of Clostridia which yield non-pathogenic spores and which are thus useful are *Clostridium acetobutylicum, Clostridium butyricum, Clostridium tyrobutyricum, Clostridium pectinovorum, Clostridium roseum, Clostridium tertium, Clostridium felsineum* and *Clostridium sporogenes*. Particular strains well suited for making the spore preparations are *Clostridium butyricum* (M-55, ATCC 13732), *Clostridium acetobutylicum* (McClung 632), *Clostridium tyrobutyricum* (McClung 1750), *Clostridium tertium* (McClung 258) and *Clostridium pectinovorum* (McClung 1188). Those organisms bearing McClung numbers are available under such numbers from the culture collection of the University of Indiana.

Although this use of Clostridial spores to achieve a significant reduction in the weight of malignant tumors is beneficial, it suffers from the disadvantage that the desired oncolysis frequently stops before the tumor is completely and permanently removed. While large parts of the tumor are destroyed, there usually remains a rim of viable tumor tissue from which regrowth can occur.

In accordance with this invention, it has been found that when tumor-bearing vertebrates are given any one or more of a number of anti-tumor chemotherapeutic drugs in conjunction with spore suspension of non-pathogenic strains of Clostridia, the reduction in tumor weight is much greater than can be realized from administration of Clostridial spores alone, or of the chemotherapeutic drug alone. In most cases the chemotherapeutic agent alone causes no significant reduction in weight of the tumor. Furthermore, the combination therapy of the invention produces a tumor weight reduction in essentially 100% of the tumor-bearing animals. In contrast, when spores alone are administered, this effect is usually found in only 70—90% of the animals even though germination of spores and some liquefaction of tumor occurs in the remaining 10—30%.

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In practicing the combination therapy of our invention, the Clostridial spores are administered to the tumor-bearing vertebrates in the same manner as when they are employed without the chemotherapeutic drug. The particular strain of Clostridium is not critical although it must, of course, be one which localizes in the tumor and causes oncolysis when used alone. We prefer to use Clostridial spore suspensions obtained from strains of *Cl. butyricum, Cl. pectinovorum, Cl. acetobutylicum* or *Cl. tyrobutyricum* although spores from the other Clostridia mentioned above are suitable.

The oncolysis and tumor weight reduction by Clostridial spores is most effective on relatively large size tumors, and for this reason the spores are normally given after the tumor has reached a significant size. In animals the tumors are allowed to grow for several days before spore therapy is begun. A growth period of 9—12 days after transplantation of Sarcoma-180 tumor in mice has been found satisfactory. After injection of spores the oncolysis and tumor weight reduction normally begins within 3—4 days and continues over a period of up to 15 days. The spores are administered suspended in a physiologically-acceptable carrier and preferably in saline solution. With the spore preparations presently in use oncolysis and weight reduction of the tumor is obtained in mice with injection of at least approximately 1×10^5 spores. However, in most cases it is preferred to use large doses in order to obtain maximal effect. These Clostridia are truly non-pathogenic and injection of large numbers of spores has no adverse effect on normal tissue.

This beneficial effect of Clostridial spores on malignant tumors is not limited to a specific type of tumor. It has, for instance, been used with success against Sarcoma-180, Ehrlich solid carcinoma and Krebs-2 carcinoma. The combination therapy of this invention gives an enhanced oncolysis and reduction in tumor weight against all tumors where the Clostridial spores produce this effect when used alone. Such tumors are hereinafter referred to as "of the type described".

In accordance with the invention, the tumor weight reducing effect obtained by administration of spores of non-pathogenic strains of Clostridia is significantly enhanced by combining such therapy with the administration of certain anti-tumor chemotherapeutic agents. Chemical agents that are highly effective in enhancing the tumor weight loss are 5-fluorodeoxyuridine (FUDR), and alkylating agents of the ethyleneimino type. Of this group of alkylating agents we prefer to employ compounds such as sec - 1 - ethyleneimino - 2-hydroxybutane (Tetramin), 2,5-bis(ethyleneimino) - 3,6 - bis - propoxy - 1,4 - benzoquinone (Bayer E-30), 2,3,5 - tris - ethyleneimino - 1,4 - benzoquinone (Registered Trade Mark: Trenimon) and Mitomycin C. Mitomycin C is considered by us as an alkylating agent of the ethyleneimino type inasmuch as it is structurally related to ethyleneimino derivatives of p-quinones. In addition to these, other alkylating agents of the ethyleneimino type are suitable for the practice of our invention. A large group of these which are known to those skilled in this art and which may be used in practicing our invention are set forth in an article by T. H. Goodridge et al, "Survey of Aziridines", Cancer Chemotherapy Reports, No. 26, page 341, January 1963, published by U.S. Department of Health, Education and Welfare.

In our studies, we have preferred to administer these chemotherapeutic agents to the tumor-bearing vertebrate intraperitoneally. However, any parenteral route is satisfactory, and intravenous or intramuscular administration could be used if desired. The drugs are normally dissolved or suspended in physiological saline, although other physiologically acceptable carriers or diluents would be suitable, e.g. aqueous glucose, Ringers solution, carboxymethylcellulose, peanut oil or mineral oil. The preferred dose level will of necessity vary with the particular compound. It is known that the alkylating agents are a group of highly toxic substances. Care should be taken to avoid administration of unduly toxic quantities of the drugs, but a certain amount of toxicity must be expected with some of the chemotherapeutic agents as a result of the dose level needed to obtain the enhancement of tumor weight reduction. With a specific chemotherapeutic agent the suitable dose levels are not difficult to determine since such drugs have been previously studied for treatment of tumors, and the preferred dose levels arrived at in those studies are also preferred in the invention. These earlier studies, however, have no other relationship to the present invention because the chemotherapeutic agents themselves have little or no effect on the tumors of the size and age which are attacked by Clostridial spores.

In order to achieve the enhanced reduction of tumor weight made possible by the invention, it is important that the drug be present in the animal body during the time of maximum oncolytic effect of said spores. The Clostridial spores may localize and germinate in the tumor, thereby causing the onset of lysis and weight reduction in 3—4 days after injection, although frequently a period of up to 9 days after injection is necessary.

Significant enhancement in tumor weight reduction has been obtained by administering the chemotherapeutic agent 1—3 days after injection of spores, or alternatively by giving drug for 1—3 days prior to spore injection in order to build up a body level, and then continuing with lower maintenance doses after injection of spores. In either case, repeated

doses of drug are administered during the period of oncolysis and tumor weight loss in order to ensure that the optimum quantity of drug is present during this process.

Experimental

In the experiments described below the spore suspensions of Clostridia are prepared in the following manner:

The cultures are maintained in the spore state by incubating for three days at 37°C. in a 22 x 175 mm. tube containing 15 ml. of Difco (Registered Trade Mark) Beef-Liver-Heart semi-solid medium. This culture can then be held at 4°C. until used for inoculum.

250 Ml. Erlenmeyer flasks containing 150 ml. of the following medium are sterilized for 17.5 minutes at 120°C.:

	Gm./Liter
Difco Meat Extract	5.0
Difco Yeast Extract	5.0
Acid Hydrolyzed Casein (Vitamin and salt free)	30.0
Sodium citrate	5.0
K_2HPO_4	2.5
Sucrose	1.5

The flasks are given a 1.5% inoculum with spores from the semi-solid medium that has been pasteurized at 80°C. for 15 minutes. The flasks are then stoppered with sterile rubber stoppers containing appropriate vents and flushed with nitrogen plus 5% CO_2. They are incubated at 37°C. for 3—4 days until there is at least 75% sporulation, then stored at 4°C. until used.

2 Liter Erlenmeyer flasks containing 1100 ml. of the same medium as described above (except that 2.0 gm./liter of sucrose are used instead of 1.5 gm./liter) are sterilized for 25 minutes at 120°C. Each of these flasks is inoculated with 20 ml. of spore inoculum from the seed flasks (prepared as above) that had been pasteurized at 80°C. for 10 minutes just prior to use. The flasks are then stoppered, flushed with nitrogen plus 5% CO_2 and incubated at 37°C. until sporulation reaches at least 90% (8—10 days). The broth from each flask is transferred aseptically to six 250 ml.-capacity sterile plastic centrifuge bottles, centrifuged for 25 minutes at 5500 rpm., and the supernatant poured off. The spores from as many as 3 flasks are packed into one set of bottles, in this manner, prior to washing. The spores in each bottle are then washed with 150 ml. of sterile, distilled, pyrogen-free water. The spores from each set of 6 bottles are then concentrated into one bottle and re-washed with 150 ml. of water as before. The entire spore crop is then suspended in 100—125 ml. of water, filtered through sterile absorbent cotton, and transferred aseptically in 1.0 ml. aliquots into sterile vials. All vials and bottles are washed with pyrogen-free, distilled water prior to sterlization. The spores are frozen in the vials and freeze-dried under vacuum for 16 hours. After lyophilization, the cotton plugs are replaced with sterile rubber stoppers. The spores are then counted.

Spores are either frozen at −20°C. in a suspension of predetermined count, or freeze-dried and kept at room temperature until resuspended in saline prior to injection.

The tumor used in the experiments was Sarcoma-180, transplanted into mice as follows:

Taconic Farms Swiss female mice weighing 16—18 gm. were used throughout. Approximately 8 x 10^6 freshly drawn cells from 7 day old Sarcoma-180 ascites tumor were suspended in 0.4 ml. of Gey's diluent and transplanted subcutaneously into the flanks of the animals. After 6 to 12 days, the mice were segregated on the basis of tumor areas obtained by measurements in two dimensions and mice bearing tumors of closely matched areas were used for each experiment. Animals were individually marked, weighed and housed into groups of six for use in the tests.

The spores suspended in 0.2 ml. of physiological saline were injected intravenously into 24 or more mice. Comparable numbers of controls were given saline alone. Other comparable groups of mice were given drug alone, and still other groups given spores and the chemotherapeutic drug. Administration of drug was the same in the groups receiving spores and those receiving no spores. At the termination of the experiment the treated and control mice were killed. The tumors were carefully dissected, freed of liquefied material by blotting, and weighed. Tumor weight in grams is reported as the average of that in surviving animals in a given group.

A. 6 x 10^7 Spores of *Cl. butyricum* in physiological saline were injected intravenously into the mice 10 days after subcutaneous transplantation of the tumor. Two days later 20 mg./kg. of 5 - fluorodeoxyuridine in physiological saline was administered intraperitoneally to groups of mice that had received the spores and to groups that had not received them. A total of seven equal doses of 5 - fluorodeoxyuridine was given over a nine day period. The average tumor weight in grams in the mice was:

Treatment	Day After Spore Therapy 11	17
1. Saline alone	4.6	5.6
2. Spores alone	1.8	3.0
3. 5-Fluorodeoxyuridine	—	4.1
4. Spores + 5-flluorodeoxyuridine	1.2	1.8

B. In an experiment carried out as in Part A, with spores of *Cl. pectinovorum*, the following results were obtained:

Treatment	Average Tumor Weight (Gms.) After Spore Therapy Day 11	Day 17
1. Saline alone	3.0	5.6
2. Spores alone	2.2	1.3
3. 5-Fluorodeoxyuridine	2.5	3.1
4. Spores + 5-fluorodeoxyuridine	1.1	0.9

C. The effect of clostridial spores and sec-1-ethyleneimino - 2 - hydroxybutane (tetramin) on tumor weight was determined. The mice were given 7 x 10^7 spores of *Cl. butyricum* (Strain M-55) intravenously 13 days after subcutaneous transplantation of the tumor. The drug was given intraperitoneally every second or third day in doses of 5 mg./kg. starting three days after spore therapy. The headings of the columns under *Average Tumor Weight* indicate day of killing after spore therapy/number of doses of drug.

Treatment	Average Tumor Weight (Grams) 9/3	13/4	16/5
1. Saline	6.4	6.7	9.6
2. Spores alone	3.8	2.7	4.5
3. Tetramin alone	3.5	4.0	3.2
4. Spores + tetramin	2.5	4.0	1.5

D. 7 x 10^7 Spores of *Cl. tyrobutyricum* were administered intravenously to mice 12 days after subcutaneous transplantation of the Sarcoma-180 tumor. Three days after spore treatment, groups of mice were given 400 mg./kg. of 2,5 - bis (ethyleneimino) - 3,6 - bis-propoxy - 1,4 - benzoquinone (Bayer E-39) intraperitoneally. Repeated doses of drug were given to these mice every other day. The headings of the columns under Average Tumor Weight indicate day of killing after spore therapy/number of doses of drug.

Treatment	Average Tumor Weight (Grams) 9/3	14/5	17/6
1. Saline	5.1	5.3	5.3
2. Spores alone	2.5	3.6	3.9
3. Bayer E—39 alone	4.4	5.0	6.9
4. Spores + Bayer E—39	3.0	3.3	1.4

E. Mice were given 9 x 10^7 spores of *Cl. butyricum* ten days after subcutaneous transplantation of Sarcoma-180. Starting one day after spore therapy 2 mg./kg. of Mitomycin C was given intraperitoneally every other day. The vertical column headings indicate day of killing after spore therapy/number of doses of drug.

Treatment	9/4 Aver. Tumor Wt. Gm.	9/4 Body Wt. Change Gm.	15/7 Aver. Tumor Wt. Gm.	15/7 Body Wt. Change Gm.
1. Saline	3.4	+1.6	5.1	+2.9
2. Spores alone	2.5	+0.5	2.3	+1.7
3. Mitomycin C alone	3.1	−2.1	2.3	−2.3
4. Spores + Mitomycin C	1.7	−0.7	0.7	−3.7

F. Sarcoma-180 was transplanted subcutaneously into mice as previously described, and the mice grouped for three separate experiments to test the effect of three dose levels of 2,3,5 - tris - ethylenemimino - 1,4 - benzoquinone (Trenimon) in combined therapy with 1 x 10^7 spores of *Cl. pectinovorum*. The spores were injected intravenously, and the drug given intraperitoneally in doses of 50, 100 and 250 μg./kg. starting three days after spore therapy. Repeated doses of drug were given as indicated below.

The headings under the column *Average Tumor Weight* indicate: day of killing after tumor transplantation/days after spore treatment/number of doses of drug.

Treatment	Average Tumor Weight (Grams)			Last Group Aver. Wt. Change In Grams	Last Group Dead/ Totla
I 250 μg/kg Trenimon	20/11/3	232/14/5	28/19/7		
1. Saline	2.3	3.8	7.4	+6.0	8/36
2. Spores alone	3.3	2.3	3.0	+5.2	9/36
3. Trenimon alone	1.7	3.2	2.0	−2.6	14/30
4. Spores + Trenimon	1.6	1.8	0.7	−5.2	25/36
II 100 μg/kg Trenimon	23/10/4	30/17/7	33/20/8		
1. Saline	3.6	4.7	3.1	+3.0	4/12
2. Spores alone	1.8	1.9	1.6	+2.4	1/12
3. Trenimon alone	3.9	5.0	3.9	−1.8	1/12
4. Spores + Trenimon	1.8	2.9	2.4	−3.5	8/13
III 50 μg/kg Trenimon	22/10/4	24/12/5	28/16/7		
1. Saline.	4.0	5.3	6.1	+1.8	1/12
2. Spores alone	1.8	2.0	2.7	+0.6	0/12
3. Trenimon alone	3.6	6.5	5.6	+0.5	1.12
4. Spores + Trenimon	1.5	1.6	2.0	−0.5	1.12

G. In this experiment the effect of administering 2,3,5 - tris - ethyleneimino - 1,4- benzoquinone (Trenimon) before spore therapy as well as after was determined. The mice were given intraperitoneally 250 μg./kg. of Trenimon on days 12, 13 and 14 after subcutaneous transplantation of Sarcoma-180. On day 15, 7×10^7 spores of *Cl. tyrobutyricum* were administered intravenously. From day 18 on the intraperitoneal administration of Trenimon was resumed at a level of 50 μg./kg. daily. The headings of the vertical columns indicate: day of killing after spore therapy/number of doses of drug after spore therapy.

	Treatment	7/4 Avg. Tumor Weight Gm.	7/4 Avg. Body Weight Change Gm.	10/7 Avg. Tumor Weight Gm.	10/7 Avg. Body Weight Change Gm.	13/10 Avg. Tumor Weight Gm.	13/10 Avg. Body Weight Change Gm.
1.	Saline	2.5	+2.3	1.7	+3.4	2.5	+2.7
2.	Spores alone	1.5	+1.7	1.2	+2.6	0.4	+1.0
3.	Trenimon alone	1.4	−2.2	0.5	−0.1	1.7	+0.1
4.	Spores + Trenimon	0.7	−3.2	0.3	−2.0	0.1	−2.4

The strains of Clostridia used in the foregoing examples were *Cl. butyricum* ATCC 13732, *Cl. pectinovorum* McClung 1188, and *Cl. tyrobutyricum* McClung 1750.

The invention is particularly applicable to warm-blooded vertebrates reared on a commercial scale. The treatment of human beings is disclaimed.

WHAT WE CLAIM IS:—

1. A method of treating tumors of the type described in vertebrates that comprises parenterally administering to such vertebrates spores of a non-pathogenic, anaerobic strain of a microorganism of the genus Clostridium capable of localizing in the tumor and causing oncolysis of the tumor, wherein there is also administered to said vertebrates as anti-tumor chemotherapeutic agent, which is 5 - fluorodeoxyuridine or an alkylating agent of the ethyleneimino type, said chemotherapeutic agent being administered parenterally to the vertebrate so as to be present in the animal body during the time of maximum oncolytic effect of the said spores.

2. A method according to Claim 1, as applied to the treatment of Sarcoma-180 tumor, Ehrlich solid carcinoma or Krebs-2- carcinoma.

3. A method as claimed in Claim 1 or 2, in which the chemotherapeutic agent is Mitomycin C, sec - 1 - ethyleneimino - 2 - hydroxybutane or 2,5 - bis (ethyleneimino) - 3,6 - bis - propoxy - 1,4 - benzoquinone.

4. A method as claimed in Claim 1, 2 or 3 in which the spores are administered 1—3 days before the chemotherapeutic agent.

5. A method according to any one of Claims 1—3, in which the chemotherapeutic agent is administered for 1—3 days before the spores to build up a concentration of the agent in the body, the administration of the chemotherapeutic agent then being continued after injection of the spores but at a lower maintenance dose.

6. A method according to any one of Claims 1—4, wherein said spores are obtained from strains of *Cl.butyricum*, *Cl.pectinovorum*, *Cl.acetobutylicum* or *Cl.tyrobutyricum*.

7. A method as claimed in Claim 6, as applied to the treatment of warm-blooded vertebrates reared on a commercial scale.

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